Development of a novel dengue virus serotype-specific multiplex real-time reverse transcription–polymerase chain reaction assay for blood screening

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BACKGROUND: Dengue fever is caused by four related RNA viruses of the genus Flavivirus, dengue virus (DENV)-1, -2, -3, and -4, which are transmitted to humans by mosquitoes. Although DENV is not endemic in Japan, an autochthonous dengue outbreak occurred in 2014. Several transfusion-transmitted cases have also been reported after the use of blood and plasma products in DENV-endemic countries. The aim of this study was to develop a novel multiplex reverse transcription–polymerase chain reaction (RT-PCR) assay for DENV blood screening.

STUDY DESIGN AND METHODS: Large-scale oligonucleotide screening was performed to obtain DENV-specific primers and probes using a variety of DENV clinical isolates. A multiplex RT-PCR assay was then developed using the identified oligonucleotides and the ability of this assay to detect DENV RNA was evaluated.

RESULTS: A number of oligonucleotides suitable for DENV RNA detection were identified and a novel DENV serotype-specific multiplex RT-PCR assay was successfully established. Comparative analysis revealed that the multiplex assay could detect levels of viral contamination as low as 100 viral copies/mL.

CONCLUSION: This established serotype-specific multiplex RT-PCR assay provides a simple, sensitive, and quantitative detection method for DENV, which could be applied in the screening of blood samples to prevent transfusion-transmitted DENV infection.

Dengue virus (DENV) belongs to the genus Flavivirus and comprises four serotypes (DENV-1, -2, -3, and -4) that are transmitted to humans by mosquitoes (Aedes sp.) in tropical and subtropical areas of the world.1 DENV infection causes a variety of diseases from asymptomatic infection and self-limited febrile illness to severe forms such as dengue hemorrhagic fever. Patients may also develop hypovolemic shock resulting from plasma leakage, which is defined as dengue shock syndrome.2 DENV infection has reemerged and increased in non-endemic areas of the world, probably as a result of the expansion of vectors in parallel with global warming and international human migration.3 Although DENV is not

ABBREVIATIONS: DENV = dengue virus; JEV(s) = Japanese encephalitis virus(-es); NIID = National Institute of Infectious Diseases.

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endemic in Japan, an autochthonous DENV outbreak that resulted in 160 cases occurred in 2014.4

In addition to vector-borne infections, there is concern regarding the risk of transfusion-transmitted DENV infection.5,6 The fact that viremic donors could be asymptomatic and therefore appear healthy raises the risk of viral contamination of donated blood. Indeed, the incidence of transfusion-transmitted DENV infection has been confirmed in multiple countries after the use of blood products including plasma, red blood cells, and platelets.7–9 Recently, Sabino and colleagues8 reported that transfusion-transmitted DENV infection could be caused by contamination levels as low as 36 to 84,400 viral copies/mL blood. Additionally, the AABB’s Transfusion-Transmitted Diseases Committee identified DENV as one of the high-priority infectious agents at risk of transfusion transmission, and they recommended blood screening for DENV.10 The development of effective blood screening assays for DENV is therefore needed to allow for routine screening, as is performed for other viruses such as hepatitis B virus, hepatitis C virus, and human immunodeficiency virus.1

Nucleic acid amplification tests (NATs) were recommended for the detection of DENV RNA by the World Health Organization dengue guidelines in 2009.1 Several reverse transcription–polymerase chain reaction (RT-PCR) assays have been developed for DENV detection and serotyping, resulting in improvements in test performance.11–16 However, one study revealed that a large number of assays developed in-house showed insufficient sensitivity and/or specificity for DENV compared with a conventional assay.17 The wide genetic diversity of DENV is a complicating factor in the establishment of NAT. However, to ensure the safety of blood products, a highly sensitive assay for DENV detection is required that is capable of detecting concentrations as low as 100 viral copies/mL.

In this study, we performed PCR-based large-scale screening of DENV-specific oligonucleotides for each serotype and identified 15 sets of novel oligonucleotides suitable for real-time RT-PCR. A multiplex real-time RT-PCR assay was then established using six sets of these oligonucleotides, designated the National Institute of Infectious Diseases (NIID) DENV 6-plex assay. This assay allowed for the simultaneous detection of all DENV serotypes in a single reaction, even in specimens containing a low viral concentration (100–1000 viral copies/mL), indicating its potential application in blood screening for DENV.

**MATERIALS AND METHODS**

**Cell culture**

African green monkey kidney cells (Vero, ATCC CCL-81) were cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific) containing L-glutamine supplemented with 10% fetal bovine serum, penicillin (50 U/mL), and streptomycin (50 mg/mL) under the temperature of 37°C and humidified 5% CO2 conditions. Vero cells were used for in vitro propagation of DENV and Japanese encephalitis viruses (JEVs).

**Specimens**

DENV clinical isolates, from dengue fever patients who returned to Japan from endemic areas, were examined for the detection and titration of the viral genome, as previously reported.11 The total numbers of clinical isolates used in this study are as follows: 12, 13, 10, and 8 strains for DENV-1, DENV-2, DENV-3, and DENV-4, respectively. Control RNAs derived from West Nile virus and yellow fever virus were purchased from VIRCELL (AmpliRun DNA/RNA controls), and JEV, strain SMB37, was propagated and collected from the culture supernatant. These viral RNA samples were employed to check the cross-reactivity of flaviviruses.

**Viral spiking**

Human plasma was spiked with three different concentrations of in vitro propagated DENV clinical isolates, with final concentrations approximately $1.0 \times 10^3$ viral copies, $1.0 \times 10^2$ viral copies, and $1.0 \times 10^1$ viral copies/mL determined by the previously reported method,11 to mimic the samples to the pooled plasma contaminated with DENV. The strains used were 01-44 for DENV-1, 01-46 for DENV-2, 00-40 for DENV-3, and 08-11 for DENV-4. These strains had no mismatched nucleotides in the regions binding to the primers and probes. Unspiked normal human plasma was used as a negative control.

**RNA purification**

Viral RNA was extracted with performed on an automated sampling instrument using a virus and pathogen mini kit (QIAasympohony SP, QIAGEN, Germantown, MD) according to the manufacturer’s instructions. The input volume for each sample was 1000 μL and the RNA elution volume was 60 μL. From the eluted RNA sample, 20 μL was transferred into a well of a PCR plate containing 30 μL of the reaction mixture and RT-PCR was performed.

**Screening of oligonucleotides for RT-PCR**

Forward and reverse primer sequences for amplification of each DENV 1-4 were designed using software (Primer Express 3.0.1, Thermo Fisher Scientific). Real-time RT-PCR was performed using a one-step RT-PCR kit (Power SYBR Green RNA-to-CT 1-Step Kit, Thermo Fisher Scientific) according to the manufacturer’s instructions. Primer sets were selected by arbitrary threshold Ct value for each serotype. TaqMan probes were designed and subjected to further screening using a one-step RT-PCR kit (TaqMan...
RNA-to-Ct 1-Step Kit, Thermo Fisher Scientific) according to the manufacturer’s instructions. Details are fully described in Appendix S1 (available as supporting information in the online version of this paper).

**DENV-specific real-time RT-PCR**

The real-time RT-PCR assay included six sets of oligonucleotide primers and five TaqMan MGB probes for DENV-1, -2, and -3 (Applied Biosystems) and a LNA probe for DENV-4 (Exiqon) as shown in Table 1. The assay could be run in singleplex (where each DENV serotype is detected in a separate reaction) or in multiplex (where all DENV serotypes are detected in the same reaction) formats designed by targeting each DENV serotype with a different colored probe (NIID DENV 6-plex assay, Table 1). All real-time RT-PCR procedures were performed and validated using a fast real-time PCR system (ABI 7500 or ABI 7500, Applied Biosystems) and a multiplex RT-PCR kit (QuantiTect, QIAGEN) according to the manufacturer’s instructions. In brief, the concentration of each primer and probe in the reaction mixture was set at 250 nmol/L for both the singleplex and the multiplex real-time RT-PCR assays. Thermal cycling variables were as follows: RT reaction at 50°C for 20 minutes, RT inactivation at 95°C for 15 minutes, and 45 cycles of denaturing at 95°C for 45 seconds followed by annealing and extension at 60°C for 45 seconds. To quantify the absolute DENV copy number in plasma specimens, in vitro synthesized and spectrophotometrically defined DENV-1 to -4 RNAs were used as standard controls in the singleplex real-time RT-PCR assay. All results were corrected by the extraction concentration factor to yield the quantity of virus present in the original plasma specimen per milliliter. The Centers for Disease Control and Prevention (CDC) DENV-1 to -4 real-time RT-PCR assay was performed in accordance with their instructions. After amplification, the threshold line was placed above the background signal, usually intersecting the initial exponential phase of the amplification curve for each serotype.

**Statistical analysis**

To determine statistical difference for positive rate between the NIID DENV 6-plex assay and the CDC DENV-1 to -4 real-time RT-PCR assay (Table 3; positive wells among eight replicates), the chi-square test for independence was performed at each viral concentration, and a p value of less than 0.05 was considered significant.

**Ethics statement**

All study procedures were approved by the ethics institutional review board of the NIID and the Japanese Red Cross Society.

### RESULTS

**Assay design**

A DENV serotype-specific multiplex RT-PCR assay was designed to detect the RNA of a wide range of clinically related strains of DENV-1 to -4 in specimens, including pooled plasma and blood products. To obtain DENV serotype-specific, sensitive primers, SYBR Green–based large-scale screening of oligonucleotides was performed using a DENV-specific primer library covering the

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**TABLE 1. List of DENV-specific oligonucleotides used in the multiplex real-time PCR assay**

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<tr>
<th>Serotype</th>
<th>Oligo name</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
<th>Start</th>
<th>End</th>
<th>Amplicon size (bp)</th>
<th>Gene</th>
<th>Dye</th>
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<td>NC_001477</td>
<td>1403</td>
<td>1425</td>
<td>68</td>
<td>Env</td>
<td>FAM</td>
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<td>1447</td>
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<td></td>
<td></td>
</tr>
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<td>1439</td>
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<td></td>
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<tr>
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<td>D1-36F</td>
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<td>FAM</td>
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<td>3131</td>
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<td>3108</td>
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<td>D2-78F</td>
<td>ACCAATGGTGCGGGCTCAT</td>
<td>NC_001474</td>
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<td>10117</td>
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<td>10142</td>
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<td>DENV-3</td>
<td>D3-16F</td>
<td>CGCGTTGATAGTGGCTTACAGACAC</td>
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<tr>
<td>DENV-3</td>
<td>D3-62F</td>
<td>GGTGGGACACAAAGAATACAGAA</td>
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<td>1689</td>
<td>76</td>
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<td>D4TEn786c-LNA†</td>
<td>CAGGATGTTA+CAGTG+CT</td>
<td></td>
<td>1723</td>
<td>1703</td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>

*Indicates that oligonucleotides have been published in Ito et al.†The LNA are represented as +nucleotides (+A/+T/+G/+C).

Start = forward primer annealing site; End = reverse primer annealing site; Gene = amplification target gene of DENV; Dye = fluorescent reporter.
complete DENV genomic sequence with the exception of frequently mutated sites: 108, 80, 72, and 71 primer sets were prepared for DENV-1, DENV-2, DENV-3, and DENV-4, respectively. According to the results of SYBR Green–based real-time RT-PCR, a total of 69 primer sets were selected because of their PCR amplification efficiencies: 17, 16, 18, and 18 primer sets for DENV-1, DENV-2, DENV-3, and DENV-4, respectively (Fig. S1, available as supporting information in the online version of this paper). From these, fluorescent TaqMan probes were designed for each set. After TaqMan-based real-time RT-PCR assays, 15 of the 69 primer sets were selected (Fig. S2, available as supporting information in the online version of this paper) and are listed in Table S1 (available as supporting information in the online version of this paper). The result of screening process was summarized in Fig. S3 (available as supporting information in the online version of this paper).

The primer sets were then analyzed for their capacity to detect a wide range of genetic variants. The 15 newly identified primer sets, along with four previously developed primer sets with a slightly modified DENV-4–specific probe,11 were subjected to PCR validation using viral RNA samples from clinical isolates of DENV-1 to -4 (Fig. S2, available as supporting information in the online version of this paper) and are listed in Table S1 (available as supporting information in the online version of this paper). The result of screening process was summarized in Fig. S3 (available as supporting information in the online version of this paper).

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In assembling the multiplex real-time RT-PCR, each probe was labeled with a distinct fluorescent dye allowing for the serotype-specific detection of DENV in a single PCR procedure. To ensure detection of all DENV-1 and DENV-3 strains, two primer and probe sets were mixed for each of these serotypes, which target different genes in DENV. The real-time RT-PCR procedures can be performed in singleplex or multiplex formats, and neither of these formats showed nonspecific signals in the absence of PCR templates.

**Assay performance and specificity**

To evaluate the performance of the singleplex and multiplex real-time RT-PCR assays, the NIID DENV 6-plex assay, the Ct values obtained by the amplification of purified viral RNA derived from 12 clinical isolates of DENV were compared. For the singleplex assays, the primer and probe sets were found to be specific for each serotype (i.e., the singleplex real-time RT-PCR for DENV-1 only detected the clinical isolates of DENV-1), but the genomes of non-DENV flaviviruses such as yellow fever virus, West Nile virus, and JEV were not detected (Table 2). For the multiplex assay, all four serotypes of DENV were detected, and no signals were observed when amplification was performed on the genomes of other flaviviruses. Ct values that were obtained in the singleplex assays specific for each serotype were equivalent to those in the multiplex assay (1.0 > ΔCt value, in Table 2), suggesting that PCR efficiency and sensitivity were comparable between these two methods. The potential cross-reactivity with nonvirus nucleic acids, including human genomic DNA and RNA derived from plasma, was also tested. The multiplex assay did not result in any nonspecific amplifications, even if high concentrations (1000 ng per reaction) or mixed DNA and RNA were used (data not shown). These results therefore indicate that our NIID DENV 6-plex assay provides specific and sensitive detection of DENV, equivalent to the singleplex real-time RT-PCR assay.
Assay sensitivity

To evaluate the sensitivity of the NIID DENV 6-plex assay, a comparative study was performed using serially DENV-spiked specimens for each serotype at three different concentrations (high, middle, and low corresponding to 1000, 100, and 10 viral copies/mL, respectively). As shown in Table 3, positive signals were detected in all reactions for DENV-1 in the NIID DENV 6-plex assay (high, 8/8 [100%]; middle, 8/8 [100%]; low, 8/8 [100%]), while the detection of those for DENV-4 was only observed at the high concentration, but neither at the middle nor at the low concentration (high, 8/8 [100%]; middle, 0/8 [0%]; low, 0/8 [0%]). Positive signals were detected in all reactions for DENV-2 and DENV-3 at both high and middle concentrations, but not at low concentration (high, 8/8 [100%]; middle, 8/8 [100%]; low, 0/8 [0%]).

For comparison with a reference assay, the CDC DENV-1 to -4 assay was performed according to the CDC recommendations. The sensitivities of both the NIID DENV 6-plex and the CDC DENV-1 to -4 assays did not differ for the detection of DENV-1 and DENV-4 (high, 8/8 [100%]; middle, 8/8 [100%]; low, 6/8 [75%] for DENV-1; high, 6/8 [75%]; middle, 0/8 [0%]; low, 0/8 [0%] for DENV-4). However, the CDC DENV-1 to -4 assay was less effective at detecting DENV-2 and DENV-3 at the lower concentrations (high, 4/8 [50%]; middle, 0/8 [0%]; low, 0/8 [0%] for DENV-2; high, 8/8 [100%]; middle, 0/8 [0%]; low, 0/8 [0%] for DENV-3). The statistical difference was observed at that point (p < 0.01, by chi-square test).

Additionally, using a virus panel consisting of RNA samples from 43 clinical isolates, the NIID assay showed lower Ct values at detecting the majority strains of DENV-2 than that of the CDC assay (Fig. S5, available as supporting information in the online version of this paper), indicating a higher sensitivity of the NIID assay relative to the CDC assay for detection of DENV-2. However, the NIID assay demonstrated similar Ct values in the detection of DENV-1, DENV-3, and DENV-4 relative to the CDC assay, indicating a comparable ability of the two assays to detect these serotypes. Furthermore, the detection limits of the newly developed multiplex assay ranged from 1 to 10 copies of each viral RNA in a single reaction, and also the assay could detect both serotypes in all combinations when mixed at equal concentrations (data not shown).

**Table 3. Sensitivity validation of the multiplex real-time PCR assay with virus-spiked plasma samples**

<table>
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<tr>
<th>Counts*</th>
<th>Serotype</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>Positive‡</th>
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<td>1000</td>
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*The number of spiked DENV copies per 1 mL of human plasma.
†The Ct values of each reaction are presented.
‡The number of fluorescent signal-positive wells among eight replicates.
ND = not detected.

**DISCUSSION**

In this study, we established a novel DENV-specific multiplex real-time RT-PCR assay that allowed for the simultaneous detection of each serotype in a single reaction. Previous studies have reported individual assays using primers and probes designed in silico to limited regions of the DENV genome including the conserved viral UTR; however, these regions might not be suitable for primer annealing, possibly resulting in impaired sensitivity or specificity. Hence, we designed a large number of primer set candidates in silico, covering the whole DENV genome, before large-scale oligonucleotide screening by real-time RT-PCR analysis. Several DENV-specific primers and probes were identified that were suitable for integration into the multiplex assay (Table 1). It is worth noting that these procedures yielded pathogen-specific oligonucleotides, which were confirmed using NAT; thus they are available for the improvement of diagnostic procedures for other emerging viruses (i.e., for Chikungunya virus or Zika virus).

It is known that DENV-infected patients—even those who remain asymptomatic—may reach concentrations as high as $10^7$ viral copies/mL blood. However, the viral concentration in blood donated from such patients may
be extremely diluted during processing of blood and plasma products; in fact, pooled plasma may contain approximately 10,000 individual donations. As mentioned, there is a need to establish a highly sensitive method for DENV screening to prevent transfusion transmission of this virus through blood products. Our assay showed positive signals with all reactions at concentrations as low as 100 viral copies/mL, indicating that the sensitivity of the assay might be sufficient for its application in blood screening (Table 3). Unfortunately, the detection limit of DENV-4 could not reach the concentration of 100 viral copies/mL, which was similar to the results obtained with the CDC DENV-1 to -4 assay. It is likely that the sensitivity of the real-time PCR assay might be affected by a combination of probe-labeled fluorescent dyes and detection platforms; thus the assay can potentially be modified as advanced dyes or systems become available.

In comparison to the CDC DENV-1 to -4 assay, our multiplex assay displayed a comparable ability to detect a wide range of DENV genetic variants (Fig. S5). Moreover, the sensitivities for detection of DENV-1 and DENV-4 with both assays were also comparable, while our multiplex assay showed greater sensitivity at detecting DENV-2 and DENV-3 (Table 3). After automated RNA extraction, our procedure may be preferable for diagnosing DENV infection and has the potential to be implemented as a blood screening system.

In addition to its potential application in blood screening, our assay could also be used for the laboratory diagnosis of DENV, which allows users to choose the singleplex or multiplex format. Because viral RNA could be detected in clinical samples obtained up to 5 to 9 days after the onset of symptoms, diagnostic assays should be supplemented with serologic analysis after the early infection period. It is anticipated that the RT-PCR assay reported here will increase the efficiency and accuracy of DENV diagnosis in both endemic and nonendemic countries.

CONFLICT OF INTEREST
The authors have disclosed no conflicts of interest.

REFERENCES
SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Appendix S1. Supplemental methods

Fig. S1. SYBR Green-based screening for DENV-specific primer library. The results of the SYBR Green-based real-time RT-PCR assay are presented. All PCR reactions were performed in duplicate, and the mean Ct value of each primer was calculated. The threshold was defined as an arbitrary Ct value (indicated by the dotted line). One bar represents one primer set. The black bars indicate the primer sets selected for further downstream screenings. ND, not detected.

Fig. S2. TaqMan-based screening for DENV-specific primer and probe sets. The results of TaqMan-based real-time RT-PCR assay are presented. All PCR reactions were performed in duplicate, and the mean Ct value of each primer was calculated. The threshold was defined as an arbitrary Ct value (indicated by the dotted line). One bar represents one primer/probe set. Black bars indicate the selected primer/probe sets that are listed in Table S1. ND, not detected.

Fig. S3. Screened oligonucleotide count. The results of the screening process are summarized. The indicated count represents the number of primer and/or probe sets.

Fig. S4. Analysis of detection of varied strains using a virus panel. Identified primer/probe sets were analyzed for detection capability using RNA specimens of 43 clinical isolates as the virus panel. Mean Ct values of each primer/probe set are represented. The primer/probe sets that showed less than the lowest Ct value plus 1 Ct in the detection of each strain are highlighted in gray. Primer/probe sets selected for real-time RT-PCR are represented as white text on a black background, and are also listed in Table 1. The four sets, D1MGBEn, D2MGBEn, D3MGBEn, and D4TEn, have been published in reference 11. VIRCELL, AmpliRun DNA/RNA Controls. ND, not detected.

Fig. S5. Comparative analysis of two assays using a virus panel. The virus panel for comparative analysis was identical to that used in Supplemental Figure 4 except for the strain, VIRCELL. Mean Ct values in each assay are represented. The delta Ct value was calculated by subtracting the Ct value of the NIID DENV 6-plex assay from the Ct value of the CDC DENV-1–4 assay.

Table S1. Screening of dengue virus-specific oligonucleotides